

# Mice divergently selected for high and low basal metabolic rates evolved different cell size and organ mass

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## Abstract

Evolution of metabolic rates of multicellular organisms is hypothesized to reflect the evolution of their cell architecture. This is likely to stem from a tight link between the sizes of cells and nuclei, which are expected to be inversely related to cell metabolism. Here, we analysed basal metabolic rate (BMR), internal organ masses and the cell/nucleus size in different tissues of laboratory mice divergently selected for high/low mass-corrected BMR and four random-bred mouse lines. Random-bred lines had intermediate levels of BMR as compared to low- and high-BMR lines. Yet, this pattern was only partly consistent with the between-line differences in cell/nucleus sizes. Erythrocytes and skin epithelium cells were smaller in the high-BMR line than in other lines, but the cells of low-BMR and random-bred mice were similar in size. On the other hand, the size of hepatocytes, kidney proximal tubule cells and duodenum enterocytes were larger in high-BMR mice than other lines. All cell and nucleus sizes were positively correlated, which supports the role of the nucleus in cell size regulation. Our results suggest that the evolution of high BMR involves a reduction in cell size in specialized tissues, whose functions are primarily dictated by surface-to-volume ratios, such as erythrocytes. High BMR may, however, also incur an increase in cell size in tissues with an intense transcription and translation, such as hepatocytes.

## Introduction

Intensity at which a multicellular body utilizes energy obtained from food, called the rate of metabolism, is a sum of energies expended by its cells. Organisms have evolved dramatic differences in metabolic rates, and undoubtedly the major determinant of this variance is body mass. Given that body mass evolves through a combination of changes in cell number and cell size (Falconer *et al.*, 1978; Stevenson *et al.*, 1995; Kozłowski *et al.*, 2010), a mass scaling of metabolic rate tells us about coevolution between cellular metabolism and body mass. Most often, we observe a decelerating increase in metabolic rate with body mass (e.g. Glazier,

2005, 2010; Makarieva *et al.*, 2008; McNab, 2008), which suggests that large organisms usually evolve cells that are metabolically less active (per cytoplasm unit) than the cells of small organisms. The mass scaling of metabolic rate differs between taxonomic groups (e.g. Glazier, 2005, 2010; White *et al.*, 2007), and it may even evolve under experimental conditions (Czar- nołęski *et al.*, 2008), indicating that organisms evolve differential coupling between their cellular metabolism and body mass. Statistical models that we fit to data on metabolic rate and body mass do not capture the entire variance in metabolic rate: equally large organisms still differ many-fold in their metabolic rates (Isaac & Carbone, 2010; White *et al.*, 2011). Thus, apparently, the evolutions of cellular metabolism and body mass can be decoupled from each other.

Despite long and hot debates on scaling laws in metabolism (Kleiber, 1947; Heusner, 1982; Kozłowski

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*et al.*, 2003; Brown *et al.*, 2004; Glazier, 2005; Maciak *et al.*, 2011; White *et al.*, 2011), the origin and evolutionary significance of mass scaling of metabolic rates still remain unclear. Emerging evidence documented links between variation in cell size and metabolic rate scaling (Chown *et al.*, 2007; Maciak *et al.*, 2011; Starostová *et al.*, 2013); however, most of recently proposed explanations of allometries of metabolic rate such as metabolic theory of ecology (Brown *et al.*, 2004), metabolic level boundaries (Glazier, 2005, 2010), dynamic energy budgets (Kooijman, 2010) still do not consider cell size as a potential factor affecting metabolic rates. According to Kozłowski *et al.* (2003) (hereafter, cell metabolism hypothesis, CMH), nonlinear changes of cell surface area with cell volume and costs associated with the maintenance of plasma membranes should lead to an inverse relation between cell size and cell mass-specific metabolic rates. Following the assumption that cell metabolic rate is completely defined by the surface-to-volume ratio, CMH predicts that organisms built from smaller cells should have higher metabolic rates (with allometric slope close to 1) than those of comparable body size, but built of larger ones (slope 2/3). However, assuming realistically that maintenance of membranes' gradients reaches roughly 20% of basic metabolic costs (Buttgereit & Brand, 1995; Hochachka *et al.*, 2003), difference between slope's values predicted by CMH must be less dramatic. What is more, we still do not know how well CMH can account for mass-independent variance in metabolic rates.

Here, we study metabolic rates and the size of five cell types in mice artificially selected for either high or low basal metabolic rate (BMR), while keeping body mass unaffected. Previous studies showed that this selection resulted in a genetically driven, 40% divergence in BMR, along with a positive sizable correlated responses in food consumption, masses of metabolically active internal organs (liver, kidney, heart and small intestine) and voluntary activity (Książek *et al.*, 2004; Brzęk *et al.*, 2007; summarized in Konarzewski & Książek, 2013). Mice from this selection experiment offer a unique opportunity to test hypotheses on coevolutionary changes in cell size and metabolism, which are decoupled from the evolution of body mass. Our primary aim is to test whether selection-driven divergence in BMR was associated with changes in cell size. Given predictions of CMH, we expect to find an inverse correlation between cell size and body mass-corrected BMR. In addition, we examined associations between cell size and nucleus size. Several lines of evidence suggest existence of a positive association between the size of a cell and the size of its nucleus, which is hypothesized to reflect links between transcription activity and nucleus size (Cavalier-Smith, 2005). A nucleus-to-cell size ratio (so-called karyoplasmic ratio) seems to play a critical

role in cell development (and so cell size) and in physiological processes in cytoplasm (Wells, 2002; Jorgensen & Tyers, 2004). Finally, we tested the hypothesis that different cell types evolve their size in concert (Kozłowski *et al.*, 2010). Note that the majority of studies that examined links between metabolic rates and cell size focused on one cell type, mainly erythrocytes, assuming a strong correlation with cell sizes of other tissues (e.g. Gregory, 2001; Maciak *et al.*, 2011). Although this assumption seems to be supported by some data (Kozłowski *et al.*, 2010), prevalence of this correlation still remains to be tested.

## Materials and methods

### Study animals

We analysed BMR, organ mass, cell size and basic haematological parameters in 20-week-old males of laboratory mice (*Mus musculus*) of six genetic lines. Two of those lines were subjected to divergent nonreplicated artificial selection either towards high (high BMR) or low (low BMR) body mass-corrected BMR (for details see Książek *et al.*, 2004). The other four lines were randomly bred as a part of a concurrent artificial selection experiment (Gębczyński & Konarzewski, 2009, 2011).

We used 30 mice from the 34th generation of high-BMR and low-BMR lines (15 from each line) and 40 mice from the 10th generation of randomly bred lines (10 from each line). Prior to our study, animals were kept individually in plastic cages at  $32 \pm 0.1$  °C and 12d:12n photoperiod with unlimited access to food (murine laboratory chow, Labofeed, Poland) and water. All procedures were approved by the local ethical committee in Białystok (approval #22/2009).

### Metabolic rate measurements

Metabolic measurements were taken between 8:00 am and 8:00 pm on animals fasted for 6 h. BMR was measured following the procedure of Książek *et al.* (2004). Briefly, we used a positive-pressure open-circuit respirometry system with dried and warmed atmospheric air pushed through the system. The airstream was divided into four streams, including one baseline, each fed to a separate mass flow controller (Sierra Instruments, Monterey, CA, USA or ERG-1000, Warsaw, Poland) forced at the rate of  $400 \text{ mL min}^{-1}$ . The system sequentially monitored metabolic rates of mice placed individually in three  $350 \text{ cm}^3$  chambers. The chambers were submerged in a water bath set at  $32 \pm 0.1$  °C (thermoneutral zone for mice). The airstream from chambers was directed to a computer-controlled channel multiplexer, a part of a Sable Systems TR-1 oxygen analyzer (Henderson, NV, USA). The air was sampled at the rate of  $75 \text{ mL min}^{-1}$ , and prior to passing through an oxygen sensor (S-3A/I Applied Electrochemistry, Pittsburgh,

PA, USA), it was scrubbed off CO<sub>2</sub> (Carboabsorb AS, BDH Laboratory Supplies, Lutterworth, UK) and moisture (Drierite, Drierite Co.LTD, Xenia, OH, USA). Each metabolic measurement trial lasted 3 h, and oxygen concentrations in each chamber were recorded every second for 2 h. We defined BMR (mL O<sub>2</sub> h<sup>-1</sup> ± 0.0013) as the lowest rate of oxygen consumption that did not change for at least 4 min by more than 0.01%. Metabolic data were analysed with a Sable System DATACAN V software. We calculated oxygen consumption rates using the formula (4a) of Withers (1977).

### Haematology and cell size measurements

After metabolic measurements, animals were weighed (g ± 0.01) and decapitated. Immediately after decapitation, blood samples were taken directly from the heart to measure haematocrit (Hct%), total haemoglobin content (Hb g dL<sup>-1</sup>), and to prepare blood smears. The smears were fixed and stained with methanol Wright-Giemsa modified solution (Sigma-Aldrich, Inc., St. Louis, Louis, MO, USA). Mice were dissected to obtain liver, kidney and duodenum for histological analyses. The liver and kidney were weighed (g ± 0.01). Skin tissue samples were taken from the dorsal side of mice. Tissue samples were fixed for 2 weeks in 4% formaldehyde, then dehydrated in ethanol and xylene, embedded in paraffin and sliced to 5 µm sections (semi-automated rotary microtome RM 2245, Leica Biosystems, Nussloch, Germany) to prepare histological slides. The slides were stained with haematoxylin and eosin.

Blood smears and histological slides were digitized under the microscope (magnification 1000×; DM 1000, Leica Biosystems, Nussloch, Germany) with a help of Leica ICC50 camera. Digital image analysis software (MultiScan Base 14.02; CSS Ltd., Warsaw, Poland) was used to measure the size of five different types of cells. We randomly chose fifty erythrocytes per blood smear and measured their diameter (µm). Assuming a circular shape of lying-flat blood cells, the diameter was used to calculate the erythrocytes' area (µm<sup>2</sup>). We measured the cross-sectional area (µm<sup>2</sup>) of hepatocytes in liver, proximal tubule cells in kidney, enterocytes in duodenum and skin epithelium cells (30 randomly chosen cells of each tissue per mouse) and the cross-sectional area of cell nuclei (µm<sup>2</sup>) by outlining the edges of cells and nuclei (areas were calculated automatically in all cases with algorithm used by MultiScan software). Data on cell and nucleus sizes were used to calculate a karyoplasmic ratio of cells (nucleus area/cell area). This index measures the relative size of nuclei. Tissue-specific mean values of cell size, nucleus size and karyoplasmic ratio were used in our hypothesis testing as characteristics of individual mice.

### Hypothesis testing

#### *Analysis of effects of selection in the absence of replicated selection lines*

Phenotypic differences between selection lines can indicate the genuine effect of divergent selection or effects of genetic drift. The method of choice for discriminating between these two possibilities is to maintain several replicated selection lines along with several control lines and test the effect of selection by a 'mixed-model' nested analysis of variance (for review see Swallow *et al.*, 2009). Despite undeniable efficacy of replication as the means for controlling random effects, many selection experiments, including ours, did not include a replicate sample (e.g. Koch & Britton, 2001; Książek *et al.*, 2004; Wislöff *et al.*, 2005). The major reason for the lack of replication in such studies is time and resource limitation. For example, in our case, it takes 4–6 weeks to collect the measurements on the sufficient number of animals to carry out the selection in just one pair of selected lines. The maintenance of another 3–4 replicates is therefore unfeasible. This is most likely why our divergent selection on BMR is the only existing one of this kind, and even though it is not fully satisfactory from a methodological standpoint, it has already provided a wealth of information (for review see Konarzewski & Książek, 2013).

To account for the shortcomings incurred by the lack of replication in our selection experiment, we applied the procedure suggested by Henderson (1997). We first calculated a magnitude of phenotypic separation ( $d_x$ ) of high-BMR and low-BMR mouse lines for each trait. Values of  $d_x$  were then expressed as multiples of intraline phenotypic standard deviations, following methods of Konarzewski *et al.* (2005). They were then compared with the magnitude of interline separation expected under genetic drift and sampling error alone (thereafter  $d_{drift}$ ), that is, in the absence of genetic correlation between the primary selected trait (BMR) and other analysed traits. Values of  $d_x$  falling within the 95% confidence intervals of  $d_{drift}$  cannot therefore be ascribed to the effect of selection. Conversely, values of  $d_x$  exceeding of  $d_{drift}$  are indicative of genetic correlations, because interline differences resulting from genetic drift should be smaller than those due to correlated response to selection on BMR (Henderson, 1997; Konarzewski *et al.*, 2005).

To estimate  $d_{drift}$ , we used equation (16) in Henderson (1997),  $d_{drift} \cong 2\sqrt{h_x^2 F + 1/n}$

where  $F$  is the coefficient of inbreeding,  $h_x^2$  is the coefficient of heritability of an analysed trait, and  $n$  is the number of families subjected to selection (22 in the case of our high-BMR and low-BMR groups). The coefficient of inbreeding  $F$  of our selection mice was estimated for the 33rd generation (0.227) using Falconer & Mackay's (1996) method. Values of  $h_x^2$  were assumed to be equal to 0.38 for BMR (Konarzewski *et al.*, 2005),

0.40 for body mass (Konarzewski, unpublished data), 0.36 for liver mass (Jones *et al.*, 1992) and 0.40 for kidney mass (Schlager, 1968). Heritability of other analysed traits remains unknown for mice (Konarzewski *et al.*, 2005); therefore, we assumed that it was fairly low ( $h^2 = 0.1$ ).

#### *Analysis of patterns of variation across all six mouse lines*

To further extend the scope of our study, we analysed variation in studied traits in a data set consisting of all six mouse lines: two lines divergently selected for BMR and the other four lines randomly bred as a part of a concurrent artificial selection experiment (Gębczyński & Konarzewski, 2009, 2011). It is important to note that although all those lines originated from an outbred Swiss-Webster strain, they were not derived from the same base population. Therefore, the randomly bred lines are not considered here as a genuine control with respect to the lines divergently selected for BMR. However, with this restriction in mind, it is still informative to carry out between-strain comparisons and infer much in the same way as with the use of comparative methods applied to different populations or species, but being free from the effect of phylogenetic inertia confounding between-species comparisons (Harvey & Pagel, 1991).

#### Statistical analyses

Variation in BMR, body mass, organ masses, haematological parameters, cell sizes and karyoplasmic ratios across all six lines was analysed with general linear model (GLM, SAS 9.1, Cary, NC, USA, 1996). The d.f. for the numerator of the *F* test was two and accounted for three fixed levels of variation (low-BMR, high-BMR and random-bred lines), whereas d.f. for denominator was three and corresponded to random variation between four nonselected lines. We used body mass as a covariate in all GLM models, except models for the haematological parameters and karyoplasmic ratios. *Post hoc* comparisons were analysed with a Tukey's test.

To examine whether cell sizes in different tissues change in concert, we calculated line-specific means of cell size in each tissue. Then we carried out a correlation analysis of means cell size in one tissue with mean cell sizes in other tissues. To examine relationships between cell size and nucleus size, we calculated line-specific mean of nucleus size and correlated them with the line-specific means of cell size.

All statistical analyses were performed on raw data as they had normal distributions.

#### Results

Table 1 reports values of separation coefficients ( $d_x$ ), which quantify the relative effect of selection on differentiation

of phenotypes between high-BMR and low-BMR lines. The separation coefficients were calculated for eight traits that according to our GLM (Table 1) diverged significantly in these two line types. All values of separation coefficient exceeded an upper limit of 95% confidence intervals for  $d_{drift}$ , which indicates a dominant role of selection in differentiation of these traits in high-BMR and low-BMR mice.

Results of GLM (Table 1) showed that the three types of lines (high BMR, low BMR and random bred) did not differ significantly with respect to body mass (Fig. 1a). Yet, we detected sizable differences in body mass-corrected BMR, with four random-bred lines having intermediate levels of BMR as compared to low-BMR and high-BMR lines (Fig. 1b). If this pattern were predominantly driven by the between-line differences in masses of metabolically active organs contributing to BMR, one would expect to find similar patterns emerging from a comparison of the masses of these organs. This was only partially supported by the GLM analyses, as mice of random-bred lines had similar liver mass to high-BMR mice, but still higher than that of low-BMR mice (Fig. 1c). On the other hand, random-bred mice had similar kidney mass to low-BMR mice but lower than that of high-BMR mice (Fig. 1d).

If the CMH holds, one would expect a close inverse match between the pattern of between-line variation in BMR (Fig. 1b) and cell sizes. In case of erythrocytes and skin epithelium cells, this inverse match was only partial, as these cell types were indeed smaller in high-BMR line than in other lines, but the cells of low-BMR and random-bred mice were similar in size (Fig. 2a and 2b, respectively). Moreover, in contrast to the CMH predictions, the size of hepatocytes, kidney proximal tubule cells and duodenum enterocytes was larger in high-BMR mice than in mice of the other lines (Fig. 2c–e).

Although we found a clear pattern of between-line type differences in erythrocyte size, it did not correspond with the differences in haemoglobin level. The amount of haemoglobin was similar between BMR-selected lines and was considerably higher in random-bred mice (Table 1). We did not find between-line differences in haematocrit.

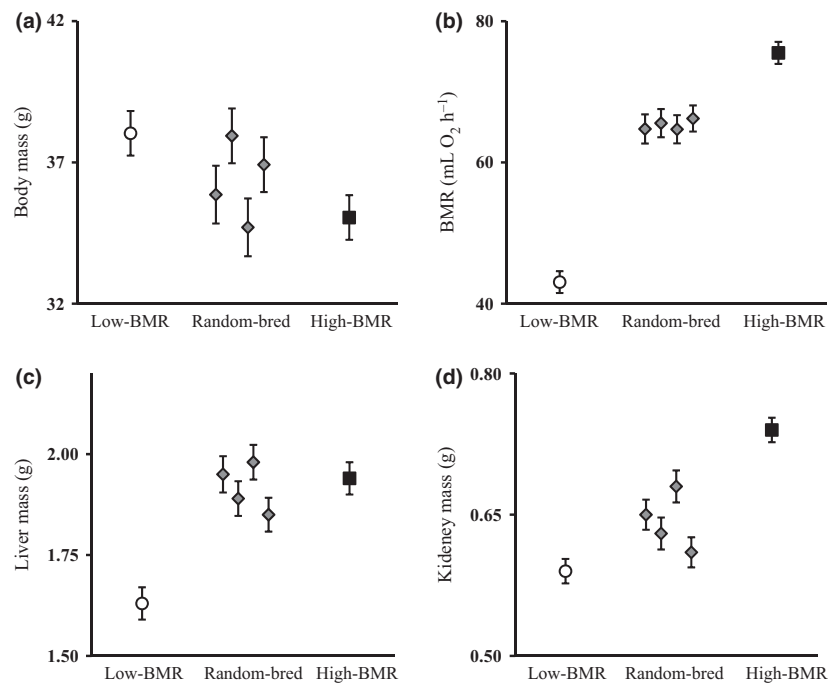
Apart from the sizes of erythrocytes and skin cells ( $r = 0.74$ ,  $P = 0.09$ ), the sizes of all other cells were significantly correlated with each other (Table 2). A positive size correlation existed between liver hepatocytes, duodenum enterocytes and kidney proximal tubule cells ( $r$  ranged from 0.93 to 0.98). The sizes of hepatocytes, proximal tubule cells and enterocytes were negatively correlated with the sizes of erythrocytes and skin epithelium cells ( $r$  ranged from  $-0.85$  to  $-0.92$ ).

All nucleated cell types had their size positively correlated with the size of nucleus ( $r$  ranged from 0.97 to 0.99; Table 2). The karyoplasmic ratio of hepatocytes in low-BMR mice was about 10% lower in comparison with high-BMR and random-bred groups (Table 1). The

**Table 1** Comparison of phenotypic traits in mice divergently selected for high or low basal metabolic rate (BMR,  $N = 15$  in both cases), and in random-bred mice ( $N = 40$ )  $\pm$  SE. Effects of line type (high BMR, low BMR, random bred) and body mass (covariate) tested with general linear models. Directional relationship with a covariate (if significant) is indicated by a sign (+ or –) together with  $P$ -values. Significant differences in mean values between line types (Tukey's test) are indicated by different letters (*abc*). The analysis of separation quantified a relative role of selection vs. genetic drift in differentiation of high-BMR and low-BMR mice. Coefficient of separation  $d_K$  measures a degree of genetic difference between the two line types;  $d_{drift}$  is an upper limit of 95% confidence interval of  $d_K$ . Values of  $d_K$  exceeding  $d_{drift}$  indicate a dominant role of selection in separation of high-BMR and low-BMR mice. Karyoplasmic ratio (KR) is a ratio of nucleus area to cell area.

Phenotypic trait	Adjusted mean value		Random bred		GLM analysis			Analysis of separation of high-BMR and low-BMR mice		
	High BMR	Low BMR			Line type		Body mass (covariate)	$d_K$	$d_{drift}$	
					$F_{2,3}$	$P$				
Body Mass (g)	35.05 $\pm$ 0.80	38.03 $\pm$ 0.80	36.40 $\pm$ 0.51		1.91	0.292	–	–	–	–
BMR (mL O <sub>2</sub> h <sup>–1</sup> )	75.52 $\pm$ 1.56 <sup>a</sup>	43.06 $\pm$ 1.55 <sup>b</sup>	65.19 $\pm$ 1.00 <sup>c</sup>		551.44	<0.001	14.28	6.101	0.726	0.726
Liver mass (g)	1.94 $\pm$ 0.03 <sup>a</sup>	1.63 $\pm$ 0.03 <sup>b</sup>	1.91 $\pm$ 0.02 <sup>a</sup>		17.44	0.022	82.48	0.909	0.713	0.713
Kidneys mass (g)	0.74 $\pm$ 0.01 <sup>a</sup>	0.59 $\pm$ 0.01 <sup>b</sup>	0.64 $\pm$ 0.01 <sup>b</sup>		10.70	0.043	31.52	2.202	0.738	0.738
Erythrocyte area ( $\mu\text{m}^2$ )	30.29 $\pm$ 0.31 <sup>a</sup>	33.27 $\pm$ 0.31 <sup>b</sup>	32.41 $\pm$ 0.19 <sup>b</sup>		17.65	0.022	0.03	2.550	0.522	0.522
Hepatocyte area ( $\mu\text{m}^2$ )	692.75 $\pm$ 7.26 <sup>a</sup>	468.64 $\pm$ 7.28 <sup>b</sup>	522.00 $\pm$ 4.52 <sup>c</sup>		80.66	0.002	1.55	7.977	0.522	0.522
Kidney proximal tubule cell area ( $\mu\text{m}^2$ )	240.04 $\pm$ 2.79 <sup>a</sup>	180.35 $\pm$ 2.80 <sup>b</sup>	200.51 $\pm$ 1.74 <sup>c</sup>		145.79	0.001	6.84	4.451	0.522	0.522
Duodenum enterocyte area ( $\mu\text{m}^2$ )	139.30 $\pm$ 1.54 <sup>a</sup>	108.65 $\pm$ 1.55 <sup>b</sup>	117.86 $\pm$ 0.98 <sup>c</sup>		61.23	0.004	16.35	4.787	0.522	0.522
Skin epithelium cell area ( $\mu\text{m}^2$ )	58.66 $\pm$ 1.18 <sup>a</sup>	71.26 $\pm$ 1.19 <sup>b</sup>	74.40 $\pm$ 0.74 <sup>b</sup>		39.49	0.007	18.41	2.729	0.522	0.522
Haematocrit (%)	42.60 $\pm$ 0.69	42.53 $\pm$ 0.69	47.08 $\pm$ 0.44		4.78	0.117	–	–	–	–
Haemoglobin (g dL <sup>–1</sup> )	13.46 $\pm$ 0.36 <sup>a</sup>	14.25 $\pm$ 0.36 <sup>a</sup>	18.97 $\pm$ 0.22 <sup>b</sup>		40.54	0.007	–	–	–	–
KR in hepatocytes	0.118 $\pm$ 0.002 <sup>a</sup>	0.107 $\pm$ 0.002 <sup>b</sup>	0.115 $\pm$ 0.001 <sup>a</sup>		4.57	0.001	–	–	–	–
KR in proximal tubule cells	0.141 $\pm$ 0.002	0.149 $\pm$ 0.002	0.145 $\pm$ 0.001		2.12	0.074	–	–	–	–
KR in duodenum enterocytes	0.182 $\pm$ 0.002	0.185 $\pm$ 0.002	0.190 $\pm$ 0.001		2.00	0.091	–	–	–	–
KR in skin epithelium cells	0.313 $\pm$ 0.005	0.306 $\pm$ 0.005	0.296 $\pm$ 0.003		2.03	0.086	–	–	–	–





**Fig. 1** Body mass (a), basal metabolic rate (BMR) (b), liver mass (c) and kidney mass (d) in lines of laboratory mice divergently selected for high BMR (black squares), low BMR (open circles,  $N = 15$  in both cases) and in four random-bred lines (grey diamonds,  $N = 40$ ). Graphs b-d show body mass adjusted means with standard errors, calculated from general linear models.

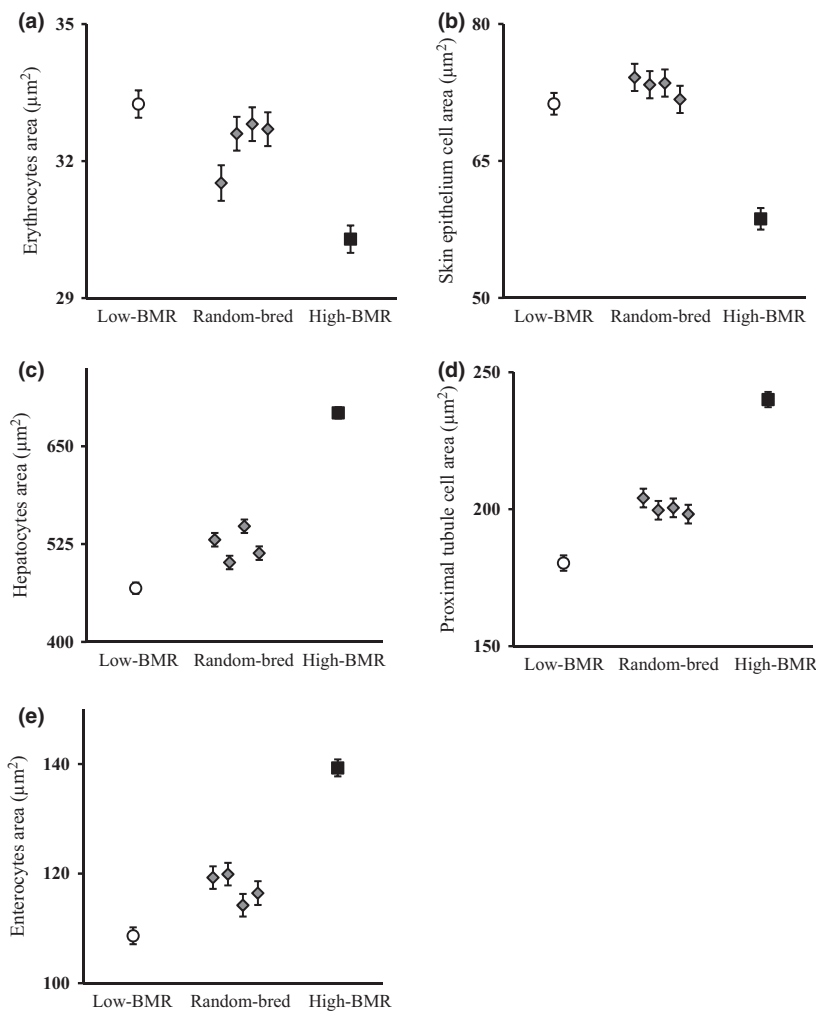
latter two groups did not differ in hepatocytes' karyoplasmic ratio. The karyoplasmic ratio of other cell types did not differ significantly between the lines of mice.

## Discussion

Our results are in agreement with the data of earlier studies demonstrating that artificial divergent selection on body mass-corrected BMR not only resulted in sizeable differences in the primary target trait, but also in a correlated divergence in mass of metabolically active organs (for review see Konarzewski & Książek, 2013). Here, we showed for the first time that this selection affected size of the cells building these organs (see Fig. 3), as well as cells of other tissues involved in metabolism, such as erythrocytes. Furthermore, the magnitude of between-line divergence in cells size was large enough to claim that they arose due to applied selection rather than genetic drift: all values of separation coefficient  $d_x$  fell outside an upper limit of 95% confidence intervals for  $d_{drift}$  (Table 1), which supports a prevailing effect of selection in differentiation of these traits between high-BMR and low-BMR mice. This suggests that changes in cell size are inherently linked to the evolution of BMR.

The above findings, however, by themselves do not demonstrate the relative contribution of the variation in cell sizes and internal organ masses to the observed within-species variation in BMR, as exemplified by the six lines of mice compared in our study. If the cell metabolism hypothesis strictly holds, one would expect

that random-bred mice having an intermediate BMR should also have intermediate size of cells. Our results, however, only partially agree with this expectation. Although our high-BMR mice were characterized by smaller size of erythrocytes and skin cells than random-bred mice and low-BMR mice, the size of these cells did not differ between the low-BMR and random-bred mice. Apparently, the attainment of low BMR involved other pathways than an increase in BMR. Such asymmetry is also evident in other cell types as well as in organ masses. Compared with other lines, the low-BMR mice had smaller kidneys and livers. Metabolism of those organs in mammals accounted for as much as 30% of the whole-body resting metabolic rate (Rolfe & Brown, 1997). Therefore, differences in the mass of these metabolically active organs are a likely explanation of the divergence of BMR in our mice. Our results, however, do not allow for disentangling the relative contribution of the size of those organs from the size of their cells on BMR. Nevertheless, the observed patterns suggest that large size of kidneys and liver of the H-BMR mice was at least partially achieved through an increase in cell size (Fig. 3). It also remains to be seen whether this mechanism accounts for an exceptionally high heart mass of H-BMR line among laboratory mice subjected to different selection regimens (Fig. 4 in Gębczyński & Konarzewski, 2011). Overall, our results are complementary to those of other artificial selection experiments, which showed that laboratory mice from lines selected for traits related to high metabolic rates have larger sizes of the internal organs compared with



**Fig. 2** Size of erythrocytes (a), skin epithelium cells (b), hepatocytes (c), kidney proximal tubule cells (d) and duodenum enterocytes (e), in lines of laboratory mice divergently selected for high BMR (black squares) ( $N = 15$  in both cases), low BMR (open circles) and in four random-bred lines (grey diamonds,  $N = 40$ ). Graph shows body mass adjusted means with standard errors calculated from general linear models.

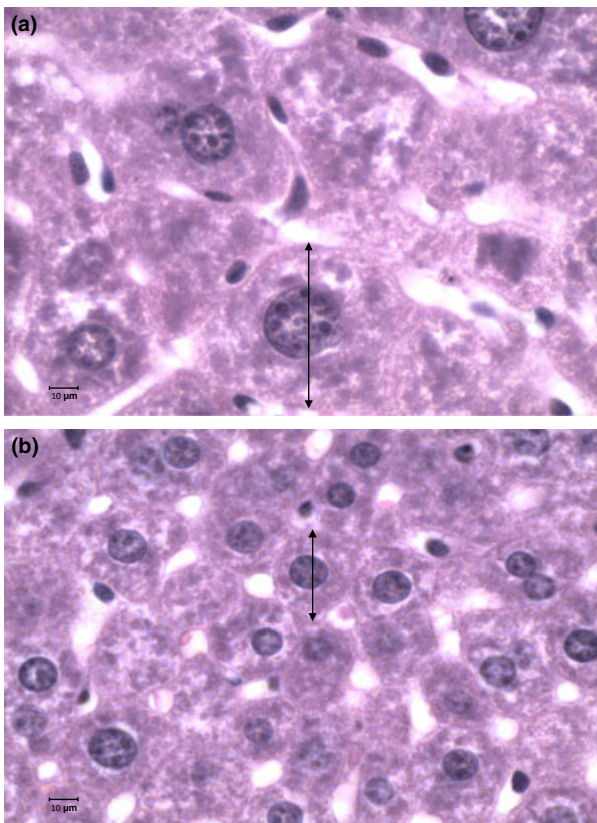
**Table 2** Correlation analysis of sizes of cells and nuclei in five tissues of mice, conducted on mean line-specific values ( $N = 6$ ; one line of high-BMR mice, one line of low-BMR mice, and four lines of random-bred mice).

	Erythrocytes	Hepatocytes	Kidney proximal tubule cells	Duodenum enterocytes	Nucleus
Hepatocytes	−0.87 $P = 0.02$				0.99 $P < 0.001$
Kidney proximal tubule cells	−0.92 $P = 0.01$	0.98 $P = 0.001$			0.99 $P < 0.001$
Duodenum enterocytes	−0.91 $P = 0.01$	0.93 $P = 0.007$	0.98 $P = 0.001$		0.97 $P = 0.002$
Skin epithelium cells	0.74 $P = 0.09$	−0.88 $P = 0.02$	−0.85 $P = 0.03$	−0.88 $P = 0.02$	0.98 $P < 0.001$

mice from lines selected for low rate of energy turnover (for review see Swallow *et al.*, 2009).

Between-line variation in erythrocyte size observed therein deserves special attention, as one can expect a particularly close link between erythrocyte size, its function and BMR. Given the central role of erythrocytes in  $O_2$  transport, the observed change in their size and number suggests that high-BMR mice evolved

higher capacity to deliver  $O_2$  to peripheral tissue. There is evidence to demonstrate that an effective exchange of  $O_2$  in capillaries requires a short axis of an erythrocyte to be about 25% larger than a capillary diameter (Snyder & Sheafar, 1999). Therefore, the size reduction in erythrocytes in high-BMR mice is also suggestive of concomitant changes in the vascular architecture of these mice. Collectively, our results suggest cellular and



**Fig. 3** Representative photomicrographs of hepatocytes and nuclei (magnification 1000 $\times$ ) in the mice selected for high (a) and low (b) BMR. Arrows indicate cell diameters.

vascular mechanisms that evolved in high-BMR mice, increasing oxidative capacity in this group. Consistent with this idea, Gębczyński & Konarzewski (2011) observed that high-BMR mice had higher peak metabolic rates during running at hypoxia than low-BMR and random-bred mice.

Large cells in livers, kidneys and duodenum of high-BMR mice, and small cells in these organs of low-BMR mice do not support the concept of CMH. This raises the question about how a change in the size of these cell types might translate into a change in the rate of metabolism. Liver and gastrointestinal tract cells of a rat in a standard metabolic state may devote about 24% and 74% of their  $O_2$  consumption to protein synthesis (Rolfe & Brown, 1997), and much of this activity serves the other cells in the body as well. The costs of service functions of all cells in an organism are estimated to constitute between 25 and 50% of the standard metabolic rate (Rolfe & Brown, 1997). Given the supracellular function of cells in livers, kidneys and duodenum, it is likely that mice evolved their BMR in tight association with shifts in the physiological service activity of these cells, and our data suggest that this activity might be linked to cell size and nucleus size.

Service functions involve a breakdown, synthesis and excretion of molecules, and this activity requires rapid transcription and translation. There is an agreement that the intensity of transcription and translation depends on the density of specific macromolecules in the nucleus and cytoplasm (Zimmerman, 1993; Ellis, 2001). Molecular crowding increases thermodynamic activities of protein synthesis machinery, but on the other hand it decreases diffusion of molecules within the cells. We propose that small cells may not be able to perform their service activity to the same degree as large cells, because biochemical reactions conducted in their small-volume cytoplasm are more prone to the dumping effect of molecular crowding. This would explain why we observed a positive association between BMR and the size of cells in organs involved in supracellular service activity. There is evidence to demonstrate that a large volume of cytoplasm requires increased rates of protein synthesis and specific genes expression (Marguerat & Bahler, 2012). An example comes from Carvalhal *et al.* (2003) *in vitro* experiments on cell cultures. Cells that were manipulated to grow larger had an increased synthesis of proteins and ATP, and an increased consumption of  $O_2$  and glucose.

The increase in translational and transcriptional activity required by larger cells was suggested to be facilitated by larger nuclei (Webster *et al.*, 2009). In support, a positive link between cell size, nucleus size and the rate of RNA transcription was reported (Schmidt & Schibler, 1995). Our data are also consistent with this idea. First, we found that nucleus size and cell size in tissues of mice were positively correlated. Second, the karyoplasmic ratio in most of these tissues did not change in association with BMR, which indicates that changes in cellular volume in these tissues were followed by a proportional change in nuclear volume. Similarly, Neumann & Nurse (2007) reported that nuclear size was proportional to cell size in fission yeast ranged over a 35-fold cell size variation. Interestingly, the karyoplasmic ratio of hepatocytes decreased its value in low-BMR mice, which indicates that low-BMR mice had hepatocytes with relatively small size of nuclei. This suggests a low transcriptional activity of a genome in these cells and agrees with the idea that the size of a nucleus matches physiological activity of a cell. An invariance of karyoplasmic ratio has long been regarded as a major puzzle in cell biology (Cavalier-Smith, 2005). Although mechanisms that control this cellular characteristic are still unclear (Cohen-Fix, 2010), our results support the view that this invariance might result from tuning of transcriptional activity to the intensity of physiological processes in cytoplasm.

Overall, it is becoming increasingly apparent that cell size cannot be ignored in studies of the evolution of metabolic rates. Emerging evidence shows that the rate of metabolism evolves in concert with cell size, and this coevolution is either coupled with evolutionary changes



in body mass (Wheatley, 2007) or, as shown by our results and Maciak *et al.* (2011), it can proceed independently of body mass. Much of this evidence shows a negative association between mass-specific metabolic rates and cell size, which complies with the concept of CMH. The crucial insight from our work is that better understanding of the coupling between the evolution of metabolic rates and cell size may require consideration of a different supracellular function and physiological activity (mainly catalytic) of cells. We envisage that the evolution of high metabolic rates might involve (i) reduction in cell size in the specialized tissues whose functions are primarily dictated by surface-to-volume ratios, such as erythrocytes; and/or (ii) the increase in cell size and thus increased intensity of translation and transcription, in the tissue with high physiological activity.

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